Original Article

Oxidative status and reproductive effort of great tits in a handicapping experiment

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Life-history theory predicts a trade-off for allocation of limited resources to reproduction and self-maintenance; however, many of the underlying physiological mechanisms remain elusive. There is growing evidence for oxidative stress to play an essential role in this trade-off because some by-products from the immune system and from normal metabolism generate reactive oxygen species that can cause oxidative damage. We manipulated reproductive effort of male and female great tits shortly before reproduction by clipping feathers of either the male or female parent of pairs of known age, given that parental effort may differ between the sexes and change over the lifetime of an individual. We quantified the effect of the treatment on morphological, physiological, behavioral, and reproductive traits. We found that feather clipping led to a decrease in parental body mass and to a reduced clutch size. Nestlings raised by clipped fathers showed reduced body mass although feeding rate was equally high between clipped and control individuals. In contrast to our predictions, we found that the feather clipping did not affect oxidative status. However, independently of the treatment, adult males had higher antioxidant capacity than females and older males showed higher oxidative damage compared with yearlings. Thus, our results suggest that the self-maintenance was prioritized over reproduction. It suggests that males are more susceptible to increased workload than females and thus more likely to reduce allocation of resources to reproduction.

Key words: **age dependence, great tits, handicapping manipulation, life-history trade-offs, oxidative status.**

INTRODUCTION

The allocation trade-off between reproduction and self-maintenance, where a greater investment in current reproduction can be achieved only at the cost to future reproduction and self-maintenance, is a classical example in life-history evolution (Stearns 1992). It has been proposed that oxidative stress, the imbalance arising when reactive oxygen species (ROS) exceed the capacity of antioxidant defense and repair mechanisms, may be important mediators of life-history trade-offs (Monaghan et al. 2009; Selman et al. 2012). ROS are by-products of normal metabolism and immune activity that may themselves directly damage the cells. In case of increased activity, such as reproduction, antioxidants are less available for self-maintenance (Harshman and Zera 2007; Nussey et al. 2009; Selman et al. 2012). Life-history theory also predicts that investment in reproduction should increase with age because the lifetime fitness costs of reproduction become smaller at lower residual reproductive value (Pianka and Parker 1975; Clutton-Brock 1984; Martin and Festa-Bianchet 2011). On the other hand, older individuals may have fewer resources to allocate to reproduction due

to physiological deterioration with aging (Kirkwood and Austad 2000).

To date, a few studies only have examined the potential relationship between elevated energy expenditure and oxidative damage, and even fewer included age effects. Increased parental oxidative stress due to higher reproductive investment was found in zebra finches (Alonso-Alvarez et al. 2006) and great tits (Losdat et al. 2011), and in fertile *Drosophila melanogaster* if compared with sterile ones (Salmon et al. 2001). Similarly, Adélie penguins with increased workload showed higher antioxidant defense but maintained equal levels of oxidative damage (Beaulieu et al. 2011). In contrast, oxidative damage in Soay sheep did not increase with age or reproductive effort but was elevated in lambs during development and growth (Nussey et al. 2009). Cold exposure, a common method to elevate energy expenditure, did not result in oxidative stress in short-tailed field voles (Selman et al. 2008) and in zebra finches (Beamonte-Barrientos and Verhulst 2013). Overall, the relationship between energy expenditure and oxidative stress remains equivocal, and studies investigating the physiological mechanisms of the trade-off between reproduction and self-maintenance are still scarce. It is assumed that the allocation of resources changes with age (Alonso-Alvarez et al. 2006). Indeed, changes in oxidative stress markers with respect to age and reproductive effort have been found in captive red-legged partridges, indicating that older

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birds show not only higher levels of oxidative damage but also higher levels of antioxidants compared with younger ones (Alonso-Alvarez et al. 2010). An analysis of long-term data on alpine swifts and great tits, 2 bird species with different life expectancies, found deterioration of cell oxidative status with increasing age (Bize et al. 2014). However, it also seems that due to selective disappearance, more resistant individuals live longer.

We experimentally increased individual workload (and potentially metabolic activity) by clipping 3 primary feathers of each wing of either the male or the female in great tit (*Parus major*) pairs shortly before reproduction. Unclipped pairs were used as controls. Feather clipping is a widespread method to induce flight and energy costs, and it significantly reduces body mass within about 1 week (Soler et al. 2008; Matysiokova and Remes 2011) and affects humoral immunocompetence (Hasselquist et al. 2001). Increased workload can affect short-term oxidative stress (Leeuwenburgh and Heinecke 2001; Costantini, Dell'Ariccia, et al. 2008; Soler et al. 2008). Individual response to increased workload may occur on morphological, physiological, behavioral, and reproductive levels, and we therefore measured effects on body mass, oxidative stress, feeding rate, clutch size, and number of fledglings. Male and female parents may pursue different reproductive strategies, and we therefore included treatment and sex in the analyses. Furthermore, we may expect energy allocation to change over individual lifetime and we thus included 3 age classes in the analyses. Individuals may respond to stressful conditions such as increased workload with regard to current reproductive effort by 1) maintenance of reproductive effort but as a consequence reduced survival, body mass, and/or elevated oxidative stress; 2) a decrease in reproductive effort but maintenance of traits favoring survival; and 3) by a reduction of both reproductive effort and survival.

MATERIALS AND METHODS

The experiment was carried out in a great tit (*P. major*) population in a forest near Bern, Switzerland (47°56′N, 7°18′E), in 2012. Nest-boxes were visited at least twice a week from beginning of March onward to monitor nest stage, start of incubation, and hatching of nestlings. Nestlings were weighed with a portable digital balance (±0.1 g) on the day of hatching and 8- and 15-day posthatch. The nestlings were ringed on day 8, and a blood sample taken and tarsus (±0.1 mm) measured on day 15. We recorded fledging success.

Feather clipping treatment

About 3 weeks before egg-laying (beginning of March), we captured males with mist nets within their territory by presentation of a great tit decoy and song playbacks of males. Females did not respond to the playbacks and were captured at night when roosting in the nest-box. Of each breeding pair, we captured either the male or female, but not both, which led to 4 treatment groups. Individuals were randomly assigned to either the clipped or control group. We then clipped the primary feathers 5, 7, and 9 on both wings to the root in experimental females ($n = 37$) and males ($n = 25$) and handled the control females ($n = 33$) and males ($n = 28$) similarly without cutting the primaries. Feathers do not regrow until the postbreeding molt (Matysiokova and Remes 2011). We measured body mass (±0.1 g), tarsus length (±0.1 mm), and third primary feather length (±0.5 mm) and ringed them if they were unringed. We aged birds according to ringing information if birds had been ringed as nestlings in previous years ($n = 94$). Unringed birds were classified as yearling breeders or older breeders based on the color of the wing

coverts (Svensson 1992) and then ringed ($n = 28$). Birds were on average 1.7 years old, median age was 1.5 years, and minimum and maximum age was 1 and 4 years, respectively. However, because sample size for 4-year-old birds was small ($n = 3$), we pooled individuals of 3 and 4 years of age in the same age category (referred to as "3+"). Great tits start breeding in their first year and reach a peak of reproductive performance when 3 years old (Bouwhuis et al. 2009). Initial body mass did not differ among treatment (*t*-test: $t = -0.01$, degrees of freedom [df] = 116.5, $P = 0.98$) and age groups (Anova: $F = 0.57$, df = 2,114, $P = 0.57$). We caught adults a second time with spring traps in the nest-box on day 12 post-hatch, first because catching female great tits during incubation and early nestling stage may induce desertion (Kania 1992), and second, because the accumulated effects of feather clipping may be strongest during this period of highest nestling food demand. We measured again body mass, tarsus, and feather length and took a blood sample (80 µL maximum) from the wing vein to later analyze the antioxidant capacity and oxidative damage from the plasma. Blood samples were kept on ice in the field (maximally 8 h), then centrifuged, and the plasma stored at −20 °C.

Parental feeding rate

Feeding rate was assessed when nestlings were 4 and 11 days old using infrared cameras (Sony HDR-CX550VE). One day before recording, the roof of the nest-boxes was fitted with a dummy camera sticker for the birds to get used to the later presence of a real camera (Coslovsky and Richner 2011). We recorded for 1.5 h and although parents usually resumed feeding a few minutes after placing the camera, we discarded the first 30 min of the video and analyzed the number of feeding visits per parent for the subsequent 60 min (Kölliker et al. 1998).

Oxidative stress analyses

Antioxidant capacity (OXY) and oxidative damage, as measured by the amount of reactive oxygen metabolites (ROMs), were quantified using the OXY-Absorbent test and the d-ROM test (Diacron International, Grosseto, Italy) following the protocols developed by Costantini and Dell'Omo (2006a) and Costantini et al. (2011). ROS are reactive macromolecules that maintain oxidizing properties such as ROMs, also called hydroperoxides that are produced in the early phase of the oxidative cascade (Costantini and Dell'Omo 2006b). The plasma (5 µL) was first diluted with 200 µL of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen and then incubated for 75 min at 37 °C. The acidic pH of the solution causes the release of metal ions from proteins, which cleave the metabolites. After reacting with an alkyl-substituted aromatic amine of the chromogen, they produce a colored complex whose intensity is directly proportional to the concentration of ROMs. After incubation, the absorbance was read with a microplate reader (PowerWave XS reader, Witec Ag, Switzerland) at 490 nm and compared with the reference curve using a control serum. The concentrations are expressed in "Carratelli units" (CARR U), where 1 CARR U corresponds to 0.08 mg of $H_2O_2/100$ mL. Hydroperoxides are expressed as millimolar of H_2O_2 equivalents. All samples were run in duplicates, which were highly correlated ($r = 0.82$, $n = 240$, $P < 0.001$).

The measured plasma antioxidant capacity is the ability of the plasma antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl), an oxidant of pathologic relevance in biological system (Costantini, Fanfani, et al. 2008). The plasma

was diluted 1:100 with distilled water. A 200-µL aliquot of HOCl solution was incubated with 2 µL of diluted plasma for 10 min at 37 °C. The same volumes were used for the calibrator and the blank. At the end of the incubation, 2 µL of the chromogen was added and the absorbance was read at 490 nm with a microplate reader (PowerWave XS reader, Witec Ag). Measurements are expressed as millimolar of HOCl neutralized according to the formula: $A_{blank} − A_{sample} / A_{blank} − A_{calibrator} × OXY_{calibrator}$, where A indicates absorbance and $OXY_{calibrator}$ is the individual concentration of the calibrator used. Results obtained using a calibration curve as a reference were qualitatively similar (Pearson $r = 0.91$, $n = 227$, $P < 0.001$) and thus not reported here. Analyses were run in duplicates, which were highly correlated ($r = 0.91$, $n = 227$, $P < 0.001$).

Ethical note

The experiment was carried out under the license BE33/12 of the Ethical Committee of the Agricultural Office of the Canton Bern, Switzerland. Bird catching and ringing were performed with permission of the Federal Agency for the Environment of the Canton of Bern, Switzerland (ringing permit 2905).

Statistical analyses

We analyzed whether the feather clipping treatment had an effect on clutch size, laying date, body mass, antioxidant capacity, oxidative damage, and nestling body mass on day 8 and 15 posthatch using linear mixed models. Analyses of posttreatment parental body mass were controlled for initial body mass as covariate. The 4 treatment groups "male clipped," "male control," "female clipped," and "female control" were analyzed by fitting the treatment (clipped or control) × sex of the treated individual. Treatment, sex, and age, fitted as a factor with 3 levels ("1," "2," and "3+"), were included as fixed factors. To estimate the influence of feather clipping on food provisioning rate and the number of fledglings, we ran generalized linear models (GLMs) using quasi-Poisson distributions due to overdispersion. Laying date and clutch size were included as covariates, and all continuous and categorical variables were centered following Schielzeth (2010). Our initial models included the 3-way interactions between treatment × sex × age and the contained 2-way interactions, and nonsignificant interactions (α > 0.05) were eliminated, starting with the highest order interaction but retaining all main effects. However, by having 10 response variables that were tested 4 times in relation to treatment, namely in the 3- and 2-way interactions and as main effect, we ended up performing 40 tests, of which 2 are expected to be significant by chance alone. Thus, without performing Bonferroni corrections, there is the risk of inflated type I errors due to multiple testing (Forstmeier and Schielzeth 2011). All analyses were performed with the free software R Version 2.15.2 (R Core Team 2013).

RESULTS

Laying date and clutch size

Laying date was independent of age ($F_{2,108} = 1.57$, $P = 0.21$), and there was no significant interaction between age and sex ($F_{2,104} = 0.84$, $P = 0.44$). Yet, there was a significant interaction between treatment and sex of the treated bird ($F_{1,108} = 6.22$, $P = 0.01$). A separate analysis of the model for males and females showed that clipped females initiated laying later ($50.3 ± 2.1$) than control-handled females ($45.8 ± 1.7$, $F_{1,59} = 3.99$, $P = 0.05$; Supplementary Table A1). In pairs where males were clipped,

laying date did not differ between treatment groups ($F_{1,47} = 1.50$, $P = 0.23$). Clutch size differed significantly among treatment groups ($F_{1,115} = 5.03$, $P = 0.03$). Control-handled birds had bigger clutch size than clipped ones (estimates ± SE: $8.52 ± 0.27$ vs. $7.90 ± 0.27$), but there was no interaction effect with sex of the treated bird ($F_{1,108} = 0.37$, $P = 0.54$). Sex of the treated bird ($F_{1,115} = 0.02$, $P = 0.90$) and age ($F_{2,116} = 0.63$, $P = 0.53$) did not influence clutch size. Separate analyses for the 2 sexes (Supplementary Material Table A2) show that clutch size of females paired with a clipped male was significantly smaller compared with those of females of control males. Clutch size of females that were clipped themselves was only nonsignificantly smaller.

Parental body mass and tarsus length

Body mass was significantly lower in clipped males and females ($F_{1,97} = 8.00$, $P = 0.01$; Table 1), and females were significantly lighter than males ($F_{1,97} = 21.22$, $P < 0.001$) on day 12 posthatch. Males that were 3 years or older were significantly heavier than yearling and 2-year-old breeders ($F_{2,97} = 3.68$, $P = 0.03$). Tarsus length was significantly different between males and females ($F_{1,104} = 62.56$, $P < 0.001$) but was not related to either treatment or age or any of the interactions (Supplementary Table A3).

Antioxidant capacity

Antioxidant capacity did not differ between clipped and control adults ($F_{1,69} = 0.22$, $P = 0.64$; Table 2). Antioxidant capacity was significantly influenced by the interaction sex × age ($F_{2,69} = 3.72$, $P = 0.03$; Figure 1). Although antioxidant capacity levels for females showed similar levels in all age classes, antioxidant capacity increased with age for males. Antioxidant capacity was higher in individuals laying later in the season ($F_{1,69} = 6.58$, $P = 0.01$).

Oxidative damage

Oxidative damage did not significantly differ between clipped and control individuals ($F_{1,79} = 2.22$, $P = 0.14$; Table 2). Oxidative damage was significantly higher in males than in females ($F_{1,79} = 6.76$, $P = 0.01$; Figure 2). It was independent of age ($F_{2,79} = 2.22$,

Table 1

Effects on body mass corrected for initial body mass using a linear mixed effect model

Effect	Estimates (±SE)	F_{df}	P
Body mass			
Intercept	16.54 (±0.15)		
Treatment[a]	−0.37 (±0.15)	$8.00_{1,97}$	**0.01**
Sex[b]	0.97 (±0.18)	$21.22_{1,97}$	**<0.001**
Age 1		$3.68_{2,97}$	**0.03**
Age 2[c]	0.08 (±0.18)		
Age 3[c]	0.37 (±0.20)		
Initial body mass	0.28 (±0.06)	$23.08_{1,97}$	**<0.001**
Laying date	−0.01 (±0.01)	$0.92_{1,97}$	0.34
Clutch size	−0.02 (±0.05)	$0.20_{1,97}$	0.66
Treatment[a] × Age[c]		*$1.84_{2,92}$*	*0.16*
Treatment[a] × Sex[c]		*$0.01_{1,92}$*	*0.92*
Age[c] × Sex[b]		*$0.98_{2,92}$*	*0.38*
Treatment[a] × Age[c] × Sex[b]		*$2.02_{2,90}$*	*0.14*

Terms eliminated from the final model are written in italics. Significant terms are written in bold.
[a]Clipped relative to control.
[b]Male relative to female.
[c]Relative to yearling breeders.

Table 2

Effects on antioxidant capacity (OXY, mM HOCl neutralized) and oxidative damage (dRom, mM H$_2$O$_2$ equivalents)

	Antioxidant capacity			Oxidative damage		
	Estimates (±SE)	F_{df}	P	Estimates (±SE)	F_{df}	P
Intercept	180.3 (±11.69)			2.55 (±0.18)		
Treatment[a]	1.79 (±12.83)	$0.22_{1,69}$	0.63	−0.13 (±0.21)	$2.22_{1,79}$	0.14
Sex[b]	−15.94 (±18.96)	$1.00_{1,69}$	0.32	0.49 (±0.21)	$6.76_{1,79}$	**0.01**
Age 2[c]	5.28 (±19.78)	$0.81_{2,69}$	0.45	−0.63 (±0.24)	$2.22_{2,79}$	0.12
Age 3[c]	−1.37 (±20.98)			−0.47 (±0.27)		
Clutch size	1.56 (±3.93)	$0.02_{1,69}$	0.89	−0.11 (±0.06)	$3.35_{1,79}$	0.07
Laying date	2.67 (±0.89)	$6.58_{1,69}$	**0.01**	−0.04 (±0.01)	$10.63_{1,79}$	**0.002**
Treatment[a] × Age[c]		*$0.03_{2,66}$*	*0.97*		*$1.58_{2,74}$*	*0.21*
Treatment[a] × Sex[b]		*$0.06_{1,66}$*	*0.80*		*$0.10_{1,74}$*	*0.75*
Sex[b] × Age 2[c]	27.63 (±30.25)	$3.72_{2,69}$	**0.03**		*$1.00_{2,74}$*	*0.37*
Sex[b] × Age 3[c]	94.95 (±34.83)					
Treatment[a] × Age[c] × Sex[b]		*$0.92_{2,64}$*	*0.40*		*$0.39_{2,72}$*	*0.68*

Terms eliminated from the final model are written in italics. Significant terms are written in bold.
[a]Clipped relative to control.
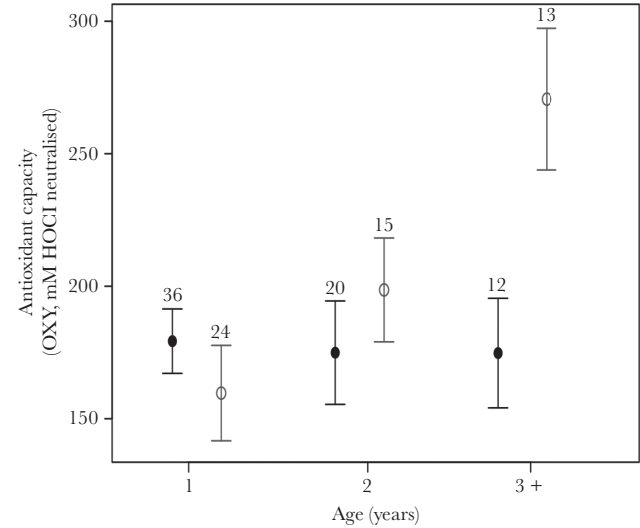[b]Male relative to female.
[c]Relative to yearling breeders.

Figure 1
Antioxidant capacity levels (model estimates ± SE) differed between males (open circles) and females (filled circles) as a function of age. Older males showed significantly higher antioxidant capacity levels than younger males, whereas antioxidant capacity levels for females were similar in all age classes. The numbers above each bar depicts the sample size for each category.

Figure 2
Oxidative damage (model estimates ± SE) was significantly higher in males than in females but was independent of the feather clipping treatment. Control-handled individuals are presented with "open squares" and clipped ones with "filled squares." The numbers above each bar depict the sample size for each category.

$P = 0.12$) and of clutch size ($F_{1,79} = 3.35$, $P = 0.07$) and significantly declined with laying date ($F_{1,79} = 10.63$, $P = 0.002$).

Feeding effort

Feeding rate on day 4 and day 11 posthatch was not affected by feather clipping treatment, age, or feeding rate of the mate and their interactions (Table 3). Brood size significantly influenced feeding rate; thus, parents adjusted feeding rate to the number of nestlings and males fed more often than females on day 4 but not day 11 posthatch (Table 3).

Nestling body mass

Nestling body mass was significantly influenced by both sex of the treated parent and the treatment as shown by a significant interaction effect both on days 8 and 15 ($F_{1,108} = 6.22$, $P = 0.01$, Figure 3
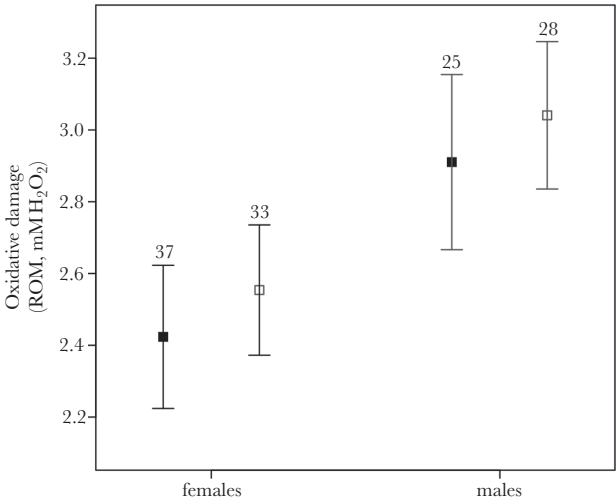
and Table 4). A separate analysis of the model for males and females showed that nestlings raised by clipped males (10.16 ± 0.31 and 13.85 ± 0.34, day 8 and day 15, respectively) were significantly lighter than nestlings raised by control-handled males (11.20 ± 0.29 and 15.54 ± 0.32). There was no significant difference between nestlings raised by clipped and control-handled females ($F_{1,52} = 0.02$, $P = 0.89$; Supplementary Table A4).

Fledging success

Number of fledglings was not significantly influenced by the feather clipping treatment ($t = -0.32$, df = 1,113, $P = 0.75$), nor by sex of the treated bird ($t = -0.32$, df = 1,113, $P = 0.75$), age 2 ($t = -0.16$, df = 1,113, $P = 0.87$), age 3 ($t = 0.44$, df = 1,113, $P = 0.66$), clutch size ($t = 0.65$, df = 1,113, $P = 0.52$), or laying date ($t = -1.81$, df = 1,113, $P = 0.07$).

Table 3

Treatment effects on food provisioning rate using a generalized linear model with quasi-Poisson distribution

|	Feeding effort day 4			Feeding effort day 11		
	Estimates (±SE)	t-Value	P	Estimates (±SE)	t-Value	P
Intercept	2.79 (±0.52)			3.67 (±0.51)		
Treatment[a]	−0.09 (±0.15)	−0.59	0.56	−0.05 (±0.09)	0.60	0.55
Sex[b]	0.47 (±0.15)	3.08	**0.003**	−0.02 (±0.09)	−0.19	0.85
Age 2[c]	−0.09 (±0.17)	−0.52	0.60	−0.003 (±0.10)	−0.03	0.98
Age 3[c]	−0.42 (±0.24)	−1.75	0.08	−0.06 (±0.12)	−0.48	0.63
Feeding rate partner	0.02 (±0.02)	1.46	0.14	0.001(±0.005)	0.25	0.81
Brood size[d]	0.12 (±0.05)	2.18	**0.03**	0.17 (±0.03)	5.59	**<0.001**
Hatching date	−0.02 (±0.01)	−1.76	0.08	−0.01 (±0.01)	−1.40	0.16
Treatment[a] × Sex[b]		*0.05*	*0.96*		*−0.34*	*0.73*
Treatment[a] × Age[c]		*0.55*	*0.58*		*−0.17*	*0.87*
Treatment[a] × Age[c]		*−0.12*	*0.91*		*<0.001*	*1.00*
Sex[b] × Age[c]		*−1.07*	*0.29*		*−0.63*	*0.53*
Sex[b] × Age[c]		*−0.31*	*0.76*		*−1.88*	*0.06*
Treatment[a] × Feeding part		*−0.80*	*0.42*		*−0.71*	*0.48*
Treatment[a] × Sex[b] × Age2[c]		*0.42*	*0.68*		*1.70*	*0.09*
Treatment[a] × Sex[b] × Age3[c]		*−1.28*	*0.21*		*0.23*	*0.82*

Terms eliminated from the final model are written in italics. Significant terms are written in bold.
[a]Clipped relative to control.
[b]Male relative to female.
[c]Relative to yearling breeders.
[d]Brood size on day 4 or day 11 posthatch, respectively.
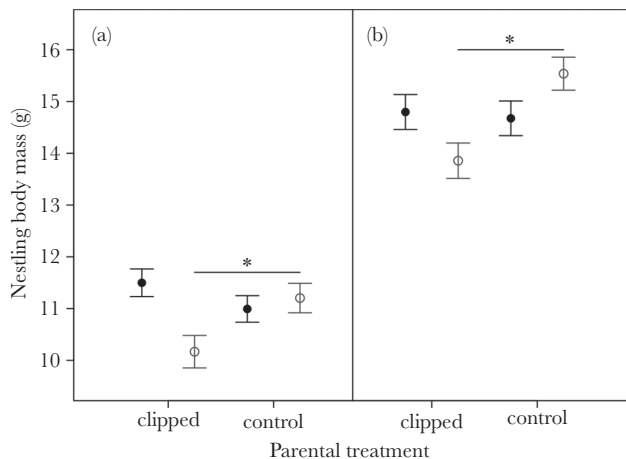
Figure 3
Nestlings raised by clipped fathers (open circles) were lighter (model estimates ± SE) than nestlings raised by clipped mothers (black circles) or by control parents. This significant interaction between treatment and sex of the clipped parent was apparent both (a) on day 8 and (b) on day 15 posthatch.

DISCUSSION

The debate whether increased energy demands and reproductive activity may compromise future reproduction and self-maintenance due to ROS production is unresolved (Selman et al. 2008; Monaghan et al. 2009). To experimentally address this question, we manipulated male and female great tits by clipping some of their primary feathers to induce increased wing load and drag, which leads to increased workload and hence potentially also oxidative stress. We found that clipping affected body mass, which demonstrates that the experimental treatment had a direct effect on the birds. Feeding rates were similar in clipped and control birds. Despite reduced body mass of clipped birds and equal level of feeding effort, and thus presumably elevated workload, there was

no effect on the levels of antioxidant capacity or oxidative damage. Studies using the feather clipping method often found a reduction of parental body mass or parental care (Winkler and Allen 1995; Sanz et al. 2000; Ardia and Clotfelter 2007; Matysiokova and Remes 2011). A possible explanation for the reduced body mass of clipped individuals is an adaptive response to the experimentally induced smaller wing surface in order to reduce wing load. Females paired to clipped males laid smaller clutches, which suggests that females adjust clutch size to the quality of their mate or expected paternal performance. Clipped females showed significantly delayed laying that may be caused by the lower energy reserves of females induced by clipping during the laying period (Williams 1996).

Relationship between oxidative stress and increased workload

Increased workload did not lead to higher oxidative damage or altered antioxidant capacity levels. Evidence for the absence of a relationship between increased workload and oxidative stress is accumulating as shown in different species (Beaulieu et al. 2011; Garratt et al. 2011; Beamonte-Barrientos and Verhulst 2013). Our prediction that increased workload may lead to increased oxidative stress, particularly with higher age, was not confirmed by the data. The relationship between metabolic rate and oxidative stress is supposedly nonlinear (Costantini 2008b) and may thus explain this result. It appears that birds have evolved unique molecular adaptations and show higher metabolic rates, and higher lifetime energy expenditure and body temperature paired with lower oxidative damage, ROS production and cell membrane saturation compared with mammals (summarized in Costantini 2008a). Greater oxygen consumption may lead, due to an uncoupling of the mitochondria, to reduced ROS production (Brand 2000; Monaghan et al. 2009; Stier et al. 2014). Alternatively, feather clipping may not be an appropriate tool to increase workload. A study in pied flycatcher (Moreno et al. 1999) and tropic house wrens (Tieleman et al. 2008) showed no significant elevation in energy expenditure in response to feather clipping, albeit with rather low sample size. A meta-analysis showed, however, that handicapped birds reduced

Table 4

Linear mixed effect model with nestling body mass on day 8 and day 15 as response variable

	Day 8			Day 15		
	Estimate (±SE)	F_{df}	P	Estimate (±SE)	F_{df}	P
Intercept	11.00 (±0.26)			14.67 (±0.31)		
Treatment[a]	0.40 (±0.37)	$0.86_{1,113}$	0.36	0.16 (±0.44)	$4.28_{1,90}$	0.04
Sex of treated parent[b]	0.17 (±0.38)	$3.39_{1,113}$	0.07	0.91 (±0.47)	$0.09_{1,90}$	0.76
Hatching date	−0.02 (±0.02)	$0.08_{1,638}$	0.78	−0.03 (±0.03)	$0.46_{1,436}$	0.50
Treatment[a] × Sex of treated parent[b]	−1.51 (±0.56)	$7.32_{1,113}$	**0.01**	−1.86 (±0.67)	$7.59_{1,90}$	**0.01**

Significant terms are highlighted in bold.
[a]Clipped relative to control.
[b]Male relative to female.

self-investment or investment in reproduction rather than elevated metabolic rate (Elliott et al. 2014). Consequently, we may expect no change of oxidative status but reduced body mass of male, female, or offspring, as found in our study, and thus explain the absence of a relationship between oxidative stress and workload. A third explanation could be that the time lapse between clipping the feathers and taking blood samples to measure oxidative stress may have been too long to observe an effect. In fact, the deleterious effect of a brood size manipulation on male oxidative stress disappeared within few days (Losdat et al. 2011).

Age- and sex-dependent effects

Overall, yearling and 2-year-old breeders were lighter than older breeders, independent of the feather clipping treatment. This might indicate a longitudinal increase of mass with age (van Balen 1967; Becker and Bradley 2007; Balbontin et al. 2012) or selective disappearance of lighter birds from the breeding population (Van de Pol and Verhulst 2006; Bouwhuis et al. 2009). Similarly, levels of antioxidant capacity were higher in older males compared with 1-year-old males. From life-history theory and more specifically the terminal investment theory, it may be predicted that young birds may rather invest in self-maintenance than in current reproduction compared with older birds and therefore would not elevate antioxidant capacity levels. A recent study found both that older alpine swifts showed higher cell resistance to oxidative stress and a trend for a similar pattern in great tits (Bize et al. 2014). An alternative explanation might be that younger breeders are lighter and show lower antioxidant capacity because resources were invested in establishing a territory (Pärt 2001). A third explanation could be that due to the age composition of our population with a higher proportion of yearling breeders, there was strong selection after the first breeding event and thus suggests that older breeders are of higher quality due to the selection process (Newton et al. 1981; Lambrechts and Dhondt 1986; Bouwhuis et al. 2009). Under this scenario, the older breeders may also have increased levels of antioxidant capacity in response to increased stress (Cohen, Hau, et al. 2008). However, age effects require cautious interpretation because population-level analyses do not necessarily provide insight into within-individual processes such as senescence (Van de Pol et al. 2006). Also here, as mentioned before, a type I error due to multiple testing cannot be excluded and one needs to await further studies.

The observed sex-specific differences in oxidative damage and levels of antioxidant capacity, except for yearling males, corroborate former findings (Marko et al. 2011; Tobler et al. 2011; Isaksson et al. 2013). High levels of antioxidant capacity might either reflect greater need for an acute response to stress (Cohen, McGraw, et al.

2008) or be a sign of individual quality as shown in collared flycatchers where males with larger forehead patches had higher levels of antioxidants (Marko et al. 2011). Finally, antioxidants may be invested into secondary sexual ornaments or eggs (Alonso-Alvarez et al. 2004) and thus lead to sex-specific effects on measures of oxidative status.

Effects on reproduction

Differential strategies across sexes might also occur in parental care (Sanz et al. 2000; Velando and Alonso-Alvarez 2003; Kokko and Jennions 2012). In both groups, fathers were feeding at higher rates than females when nestlings were 4 days old, as expected given that females need to keep nonthermoregulating nestlings warm most of the time. Nestlings raised in nests where the father was clipped were lighter than nestlings raised in nests with clipped mothers or control parents, despite equal feeding rates among treatment groups. It is feasible that clipped fathers were less selectively foraging for food in the near surrounding and thus bringing poorer food, eventually leading to lighter nestling body mass at later ages due to a carryover effect. It has been found previously that an increase in feeding rate does not necessarily imply an increase in effective food provisioning because it could just mean that the delivered food is qualitatively poorer (Wright et al. 1998; Sanz et al. 2000). Later, females were feeding at equal rate and thus may have compensated for the poorer performance of their male partner. The reduced nestling body mass on day 8 and day 15 may also be a carryover effect of the earlier phase when the male mainly provisioned food. Although the number of fledglings was not affected by either treatment or sex of the clipped parent, offspring survival is strongly affected by the body mass at fledging in small passerines (Tinbergen and Boerlijst 1990), and thus, moderate reductions in parental investment during offspring rearing, as shown in our study, can likely incur substantial fitness costs. Moreover, males seem to be more susceptible to stressful conditions and might be more willing to sacrifice offspring, in addition to the lower level of confidence in paternity (Slagsvold and Lifjeld 1990; Sanz et al. 2000).

CONCLUSIONS

We predicted 3 different scenarios: the first one predicted an investment in current reproduction with costs on self-maintenance and survival, whereas the second one predicted a reduced investment in reproduction and maintenance of traits favoring survival and the third one favored self-maintenance up to a certain level with intermediate costs for current reproduction. Our results correspond most closely to the third scenario. There are indications that the feather clipping treatment did impair parents by reducing body

mass while these individuals kept feeding rate at a constant level. On the cellular level, there was no evidence that clipped individuals suffered more than control-handled individuals. Thus, it seems that females maintain offspring quality at the expense of self-maintenance, whereas males distribute the costs between themselves and the offspring.

SUPPLEMENTARY MATERIAL

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